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# Inducible Phenotypic Multidrug Resistance in the Fungus *Mucor racemosus*†

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**The dimorphic fungus *Mucor racemosus* exhibited a single-step, inducible resistance to cycloheximide, trichodermin, and amphotericin B. Cells adapted to inhibitory levels of the antibiotics after 12 to 40 h. The adaptation involved all the cells in the population and was not the result of the selection of resistant mutants. Adaptation to one drug provided cross resistance to other, dissimilar drugs. Resistance was lost within several generations of growth in the absence of the inhibitors.**

Resistance to drugs and fungicides presents an ever-increasing problem in the clinical and agricultural control of fungi. While mutational resistance is an obvious suspect in cases of fungal persistence, numerous reports have established the phenomenon of phenotypic (epigenetic) resistance or adaptation to drugs by fungi in the laboratory (3, 4, 10, 15, 16). Phenotypic adaptation has been recognized in a wide variety of fungi, including the zoopathogens *Trichophyton mentagrophytes* (5), *Cryptococcus neoformans* (2), and *Candida albicans* (3, 4, 17) as well as the phytopathogens *Sclerotinia fructicola* (11) and *Ustilago maydis* (7). A broad range of inhibitors was included in these studies, including antibiotics, antimetabolites, and synthetic fungicides.

In most reports, phenotypic resistance was developed by serial transfer and selection in increasing drug concentrations. Since this regimen doubtlessly leads to many genetic and regulatory alterations, it presents apparent difficulties to controlled studies of the adaptation mechanism. Single-step adaptation (spontaneous population recovery from an initially inhibitory drug challenge) has been reported for a single inhibitor of *U. maydis* (7) and in subpopulations of the genus *Candida* (17).

For several years we have used cycloheximide or the mycotoxin trichodermin to inhibit amino acid incorporation into proteins of *Mucor racemosus*. However, when several hundred spores were placed on solid medium containing these drugs, all grew to form colonies. As this report shows, after an initial period of susceptibility to these antibiotics, all the cells in the population acquire a phenotypic resistance to them. Moreover, cells which have adapted to one inhibitor become resistant to others.

## MATERIALS AND METHODS

*M. racemosus* (*Mucor lusitanicus* ATCC 1216B) was employed in all adaptation studies. The growth medium was composed of 0.5% peptone, 0.05% yeast nitrogen base (both from Difco Laboratories, Detroit, Mich.), and 2.0% glucose. Growth of the yeastlike form of *M. racemosus* was monitored with a Klett-Summerson colorimeter and a green filter (540 nm).

Cycloheximide was purchased from Sigma Chemical Co., St. Louis, Mo. Amphotericin B was from E. R. Squibb and Sons, Princeton, N.J. Trichodermin was the kind gift of W. O. Godtfredson, Leo Pharmaceutical Products, Ballerup, Denmark.

Autoradiography was performed as previously described (13). Cell samples were removed from the culture at intervals. [$^3$H]leucine was added to the sample, and incubation was continued in parallel with the parent culture. Cells were then washed, applied to microscope slides, and fixed. The slides were coated with photographic enulsion and exposed for 4 days; they were evaluated by counting the number of grains in 100 cells and all of the cells in several fields.

## RESULTS

By following the kinetics of growth, it was found that *M. racemosus* was initially susceptible to three antifungal antibiotics: cycloheximide, the mycotoxin trichodermin, and amphotericin B. However, we discovered that over longer periods of time (typically 1 to 2 days), *M. racemosus* exhibited spontaneous phenotypic recovery from inhibition by these inhibitors.

**Kinetics of drug adaptation.** Drug adaptation took place in either the hyphal or yeastlike morphology of *M. racemosus*, during hyphal morphogenesis, and spore germination. For quantitative kinetic studies we chose to follow the growth and adaptation of the yeastlike cultures and take advantage of their uniform exponential growth and ease of handling. Growth curves of yeastlike *M. racemosus* cells grown under 100% $CO_2$ are presented in Fig. 1. The culture was divided at zero time and treated with either 100 μg of cycloheximide, 5 μg of trichodermin, or 0.4 μg of amphotericin B per ml. In all cases, net growth ceased soon after drug addition, and cells underwent a lag period before growth resumed. This lag was about 25 h for cycloheximide or trichodermin, but was often as long as 40 h for amphotericin B. After the lag, exponential growth resumed at a reduced rate characteristic of the drug employed. The ultimate growth potential of both cycloheximide- and trichodermin-treated cultures was somewhat depressed.

**Colony plating studies.** Under 100% $CO_2$ *M. racemosus* forms discrete yeastlike colonies on solid medium. When colonies were patched or replicated onto plates containing inhibitory concentrations of either cycloheximide, trichoder-

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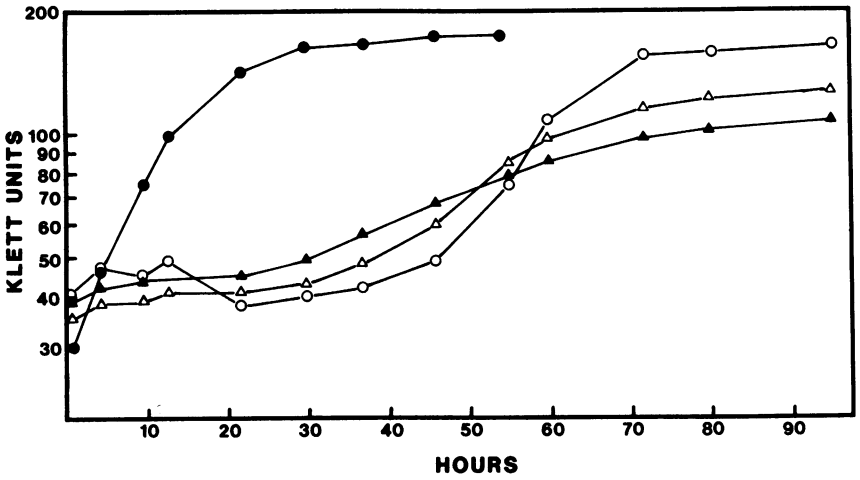


FIG. 1. Kinetics of drug adaptation. An early exponential culture was split at zero time and challenged with drugs. Symbols: ●, control culture (no added drugs) ▲, cycloheximide, 100 μg/ml; △, trichodermin, 5.0 μg/ml; ○, amphotericin B, 0.4 μg/ml.

min, or amphotericin B, 100% of the original colonies arose after a lag period. These colonies were adapted, in that they gave rise to colonies more quickly than unadapted or naive colonies upon subsequent transfer to fresh drug plates. Direct plating efficiencies on either trichodermin or amphotericin B plates (CFU from liquid culture cells compared with nondrug plates) were also 100%. Plating efficiencies for cycloheximide plates were somewhat lower, from 25 to 70% depending on medium and drug level. This appears to be an effect of plating on solid medium.

**Autoradiography of drug-adapting cells.** The adaptation to cycloheximide of individual cells in liquid culture was tested by autoradiography as a direct assay of protein synthesis in individual cells. Samples were taken at intervals before treatment with cycloheximide, during the adaptive lag period, and after the resumption of growth. All of the cells in a field resumed protein synthesis after the adaptive lag (Fig. 2). This result argues against the possibility that adaptive growth was the result of selecting a resistant population of cells.

**Adaptation by prior exposure to inhibitors.** The lag period of growth observed at high drug concentrations (Fig. 1) might represent a synthetic period during which cellular components accumulated to levels adequate to cope with the drug challenge. Alternatively, the adaptive mechanism might function constitutively; in this case, lag periods would simply reflect the time required to gradually overcome a given drug level (e.g., by transport out of the cell). As a means of understanding the mechanisms of adaptation, cells were first grown in low levels of either cycloheximide, trichodermin, or amphotericin B and then challenged with high levels of the same drugs. These low drug levels reduced the growth rates slightly but did not produce a growth lag. Cells propagated in low levels of cycloheximide and trichodermin were found to be resistant to these compounds when exposed to higher drug levels (Fig. 3). Pretreatment of cells with low levels of amphotericin B generally protected the cells against the inhibitory effects of higher concentrations of the antibiotic. However, this result was less pronounced than results of pretreatment with cycloheximide and tricho-

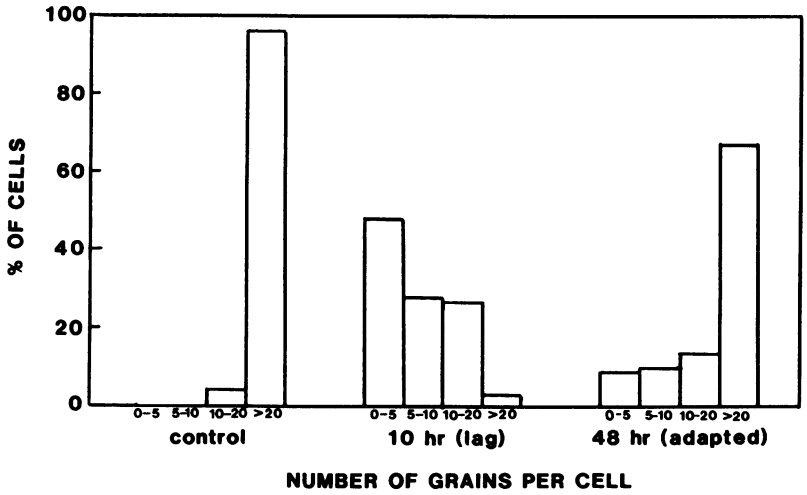


FIG. 2. The number and distribution of photographic grains in autoradiographic micrographs of *M. racemosus*. The percentage of the population showing that number of grains is shown on the ordinate. The method of labeling, fixation, and autoradiography was that of Orlowski and Sypherd (13).

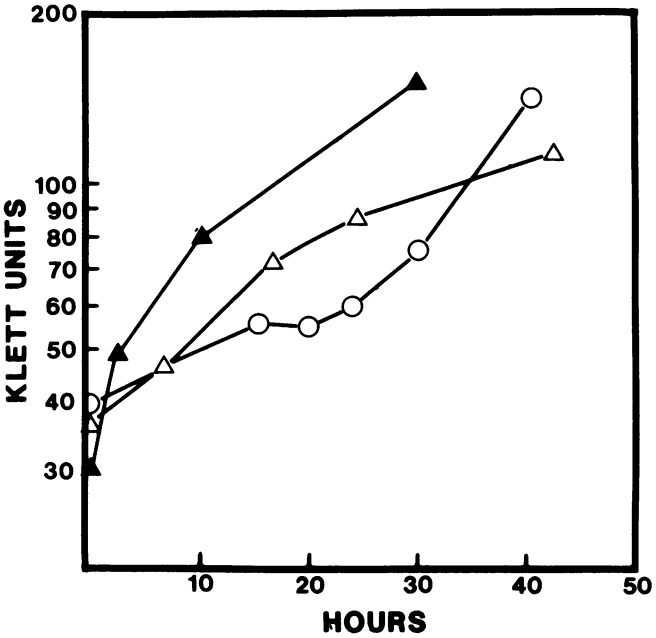


FIG. 3. Induction of drug adaptation. Early exponential cultures growing in low levels of drugs were supplemented at zero time with additional drugs. Symbols: ▲, culture growing in 20 μg of cycloheximide per ml, increased to 100 μg/ml; △, culture growing in 0.625 μg of trichodermin per ml, increased to 5.0 μg/ml; ○, culture growing in 0.0625 μg of amphotericin B per ml, increased to 0.4 μg/ml.

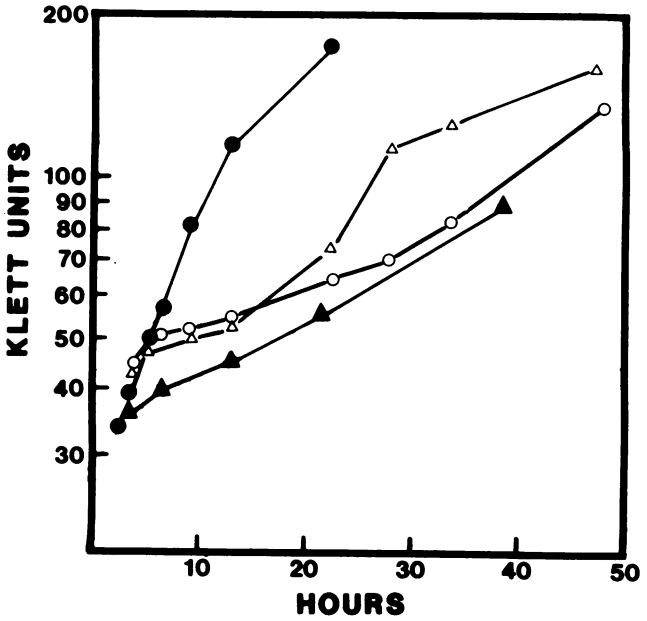


FIG. 4. Cross adaptation by cells adapted to cycloheximide. Early exponential cells growing in 100 μg of cycloheximide per ml (adapted to cycloheximide) were washed and immediately tested for resistance to fresh drugs. Symbols: ●, control culture (no added drug); ▲, cycloheximide, 100 μg/ml; △, trichodermin, 5.0 μg/ml; ○, amphotericin B, 0.4 μg/ml.

dermin (Fig. 3). Despite the variable growth response to amphotericin B, it seems clear that preadaptation by exposure to low drug concentrations abolished the drug lag period characteristic of an initial high-level drug challenge. Resistance therefore appears to be inducible, and high levels of resistance may derive from synthesis, accumulation, or assembly of novel cellular components.

**Multidrug resistance and cross adaptation.** To test the possibility that a single mechanism controls adaptive resistance, cultures adapted to a single drug were tested for cross resistance to other drugs. Cells adapted to cycloheximide were harvested, washed, and divided among four test cultures (Fig. 4). A drug-free control culture quickly resumed an uninhibited growth rate, demonstrating that adaptation does not result in drug dependency. A second control culture was immediately rechallenged with fresh cycloheximide. This sample continued to grow at the adapted growth rate characteristic of the cycloheximide concentration. This result indicates that adaptation is not simply the result of complete inactivation of cycloheximide in the culture medium. Test cultures treated with either trichodermin or amphotericin B failed to show typical drug lags of 20 to 30 h and assumed drug-specific adapted growth rates. Thus, cycloheximide either induced a common adaptive mechanism providing resistance to all three drugs or acted as a common inducer of several mechanisms.

In an analogous experiment (Fig. 5), trichodermin-adapted cells were tested for cross resistance. As with cycloheximide-adapted cells, control cultures showed that drug dependency was not associated with trichodermin adaptation. Test cultures showed immediate resistance to both cycloheximide and amphotericin B. Therefore, trichodermin, like cycloheximide, was also a common inducer of an adaptive resistance.

Cells adapted to amphotericin B were also tested for cross resistance to cycloheximide and trichodermin (Fig. 6). Again, control cultures ruled out drug dependency. In contrast to results obtained with cycloheximide- and trichodermin-adapted cells, amphotericin B-adapted cells showed suscep-

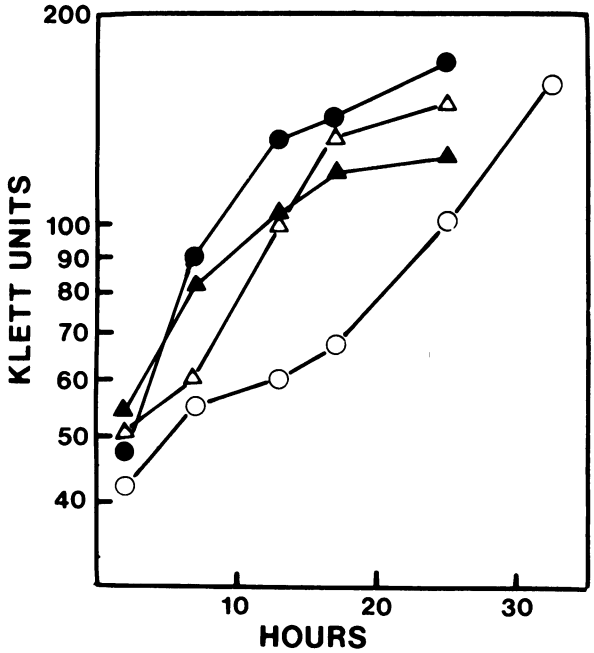


FIG. 5. Cross adaptation by cells adapted to trichodermin. Early exponential cells growing in 5.0 μg of trichodermin per ml (adapted to trichodermin) were washed and immediately tested for resistance to fresh drugs. Symbols: ●, control culture (no added drug); ▲, cycloheximide, 100 μg/ml; △, trichodermin, 5.0 μg/ml; ○, amphotericin B, 0.4 μg/ml.

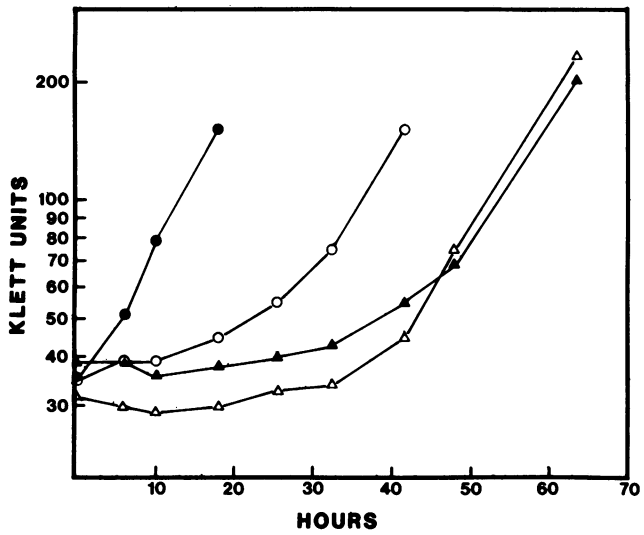


FIG. 6. Cross adaptation by cells adapted to amphotericin B. Early exponential cells growing in 0.4 μg of amphotericin B per ml (adapted to amphotericin B) were washed and immediately tested for resistance to fresh drugs. Symbols: ●, control culture (no added drug); ▲, cycloheximide, 100 μg/ml; △, trichodermin, 5.0 μg/ml; ○, amphotericin B, 0.4 μg/ml.

tibility to cycloheximide and trichodermin typical of unadapted cells. Complete reciprocity therefore did not exist with respect to cross adaptation among drugs.

**Deadaptation or resensitization to drugs.** By definition, phenotypic resistance is unstable and is lost in the absence of a drug. Replica plating experiments confirmed that resistance was lost upon colony transfer to drug-free solid medium. Quantitation of drug deadaptation was performed in liquid cultures with $CO_2$-grown yeastlike cells.

Cells were first adapted to trichodermin (5 μg/ml), amphotericin B (0.4 μg/ml), or cycloheximide (100 μg/ml), then washed free of the antibiotic, and resuspended in fresh growth medium. At intervals, samples were taken from the culture and challenged with one or more antibiotics. In this way a determination was made of the original growth period required for the cells to regain their susceptibility to the various inhibitors. Table 1 summarizes these results. After as few as 1.5 doublings in the absence of trichodermin, the cultures acquired the same degree of susceptibility to trichodermin, cycloheximide, and amphotericin B as before they became adapted. Similarly, amphotericin B-adapted cells quickly assumed their susceptibility to all three drugs, becoming fully susceptible after 2.1 cell doublings.

In contrast to the results with trichodermin and amphotericin B, cells adapted to cycloheximide were slow to recover drug susceptibility. More than 15 doublings in a drug-free medium were required for the cells to become fully susceptible challenge by cycloheximide (Table 1). Cycloheximide-adapted cells were also slow to recover their susceptibilities to the other two drugs (data not shown).

The deadaptation of cells to the three drugs suggests that some cellular components responsible for resistance were diluted out or turned over as the cells grew. The more gradual acquisition of susceptibility of cycloheximide-adapted cells may imply a different mechanism, but we suspect that the longer period of adjustment reflects the dilution of intracellular cycloheximide as well as the cellular components that impart resistance. High levels of cycloheximide (100 μg/ml) were used for the adaptation studies, but as little as 4 μg/ml induced the resistance mechanism. Although the concentration of cycloheximide would be reduced to about 3 μg/ml after six generations, additional growth may be required to reduce the level of cellular components that control the resistance process.

## DISCUSSION

Adaptation to inhibitors has been reported in cultured mammalian and plant cells (1, 6, 8, 12) and in protozoa (9, 14, 18). Although initially susceptible to cycloheximide, trichodermin, and amphotericin B, cells of *M. racemosus* adapted to these antibiotics after several hours. This adaptation involved all the cells in the population, with the result that the entire culture became resistant to the drugs. Despite their dissimilar structures and modes of action, adaptation to either cycloheximide or trichodermin results in cross resistance to the other two antibiotics. Therefore, the mechanism of this adaptation to dissimilar antifungal agents is not easy to assess. An analysis of the inducibility and the resensitization to drugs suggests that novel cellular components are correlated with drug adaptation. Moreover, the patterns of cross adaptation suggest that the resistance mechanism consists of several components. A determination of the mechanism would be greatly facilitated by the isolation of mutants with altered responses to the drugs. We have been successful in isolating mutants which are constitutively resistant (i.e., they do not show the initial period of inhibition by the drug) as well as mutants that never adapt to the antibiotics. In our preliminary studies with radioactive trichodermin, we found that acquired resistance to this antibiotic was not due to a decrease in uptake of the drug. We are presently concentrating our studies on mechanisms that would lead to the modification of the antibiotics.

A study of the adaptation phenomenon reported here may

TABLE 1. Deadaptation of *M. racemosus* to antibiotics and acquisition of drug susceptibility

| Cells adapted to: | No. of cell doublings without antibiotic | Challenged with: | Growth lag (h) in presence of inhibitors[a] |
|---|---|---|---|
| None | | None | 0 |
| | | Cycloheximide | 25 |
| | | Trichodermin | 25 |
| | | Amphotericin B | 40 |
| Trichodermin | 1.5 | None | 0 |
| | | Cycloheximide | 25 |
| | | Trichodermin | 20 |
| | | Amphotericin B | 40 |
| Amphotericin B | 2.1 | None | 0 |
| | | Cycloheximide | 25 |
| | | Trichodermin | 25 |
| | | Amphotericin B | 25 |
| Cycloheximide | 4.5 | None | 0 |
| | | Cycloheximide | 0 |
| | 10 | Cycloheximide | 0 |
| | 12.8 | Cycloheximide | 20 |
| | 15.5 | Cycloheximide | 25 |

[a] Growth lag before the resumption of growth during the challenge period. Cells were adapted to inhibitor and then grown in drug-free medium for the period indicated. Nonadapted cells grown with inhibitors had growth lags of 26 h in cycloheximide, 25 h in trichodermin, and more than 40 h in amphotericin B.

provide important new insights into the generally resistant nature of fungi. It is also possible that the acquired resistance to amphotericin B has clinical significance in the treatment of fungal infections. Whatever the mechanism of the adaptive resistance, its elaboration could reveal interesting biochemical paradigms in microbial detoxification of mycotoxins and other inhibitors.

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## LITERATURE CITED

1. **Adair, G. M., and J. H. Carver.** 1979. Unstable, non-mutational expression of resistance to the thymidine analogue, trifluorothymidine in CHO cells. Mutat. Res. **60**:207–213.
2. **Bodenhoff, J.** 1968. Development of strains of *Cyptococcus neoformans* resistant to nystatin, amphotericin B, trichomycin and polymyxin B. Acta Pathol. Microbiol. Scand. **73**:572–582.
3. **Capek, A., L. Pavlikova, A. Simek, L. Bruna, U. Janata, and Z. Budesinsky.** 1973. Antimicrobial agents. XIX. Detoxification mechanisms of some strains of *Candida*. Folia Microbiol. **18**:522–523.
4. **Capek, A., A. Simek, and J. Keyda.** 1972. Antimicrobial agents. XVIII. Inducible resistance in species of the genus *Candida*. Folia Microbiol. **18**:304–307.
5. **Capek, A., A. Simek, and J. Leiner.** 1970. Antimicrobial agents. VI. Antimycotic activity and problems of resistance. Folia Microbiol. **15**:314–317.
6. **Davies, M. E., and C. P. Exworth.** 1973. Transient inhibition by cycloheximide of protein synthesis in cultured plant cell suspensions: a dose response paradox. Biochem. Biophys. Res. Commun. **50**:1075–1080.
7. **Esposito, R. E., and R. Holliday.** 1964. The effect of 5-fluorodeoxyuridine on genetic replication and mitotic crossing over in synchronized cultures of *Ustilago maydis*. Genetics **50**:1009–1017.
8. **Fox, M., and M. Radacic.** 1978. Adaptational origin of some purine-analogue resistant phenotypes in cultured mammalian cells. Mutat. Res. **49**:275–296.
9. **Frankel, J.** 1970. An analysis of the recovery of *Tetrahymena* from effects of cycloheximide. J. Cell. Physiol. **76**:55–64.
10. **Griffin, D. H., S. B. Sullia, and I. F. Salkin.** 1978. Resistance of selected saprobic and zoopathogenic fungi to cycloheximide. J. Gen. Microbiol. **105**:127–134.
11. **Grover, A. K., and J. D. Moore.** 1981. Adaptation of *Sclerotinia fructicola* and *Sclerotinia laxa* to higher concentrations of fungicides. Phytopathology **51**:399–401.
12. **Harris, M.** 1979. Genetic and adaptive differences in the expression of drug resistance in hybrid cells. Somatic Cell Genet. **5**:793–808.
13. **Orlowski, M., and P. S. Sypherd.** 1978. Location of protein synthesis during morphogenesis of *Mucor racemosus*. J. Bacteriol. **133**:399–400.
14. **Roberts, C. T., and E. Orias.** 1974. On the mechanism of adaptation to protein synthesis inhibitors by *Tetrahymena*. Facilitation, cross adaptation, and resensitization. J. Cell Biol. **62**:707–716.
15. **Salkin, I. F.** 1975. Adaptation to cycloheximide: *in vitro* studies with filamentous fungi. Can. J. Microbiol. **21**:1413–1419.
16. **Salkin, I. F., and N. Hurd.** 1972. Quantitative evaluation of the antifungal properties of cycloheximide. Antimicrob. Agents Chemother. **1**:177–184.
17. **Sarachek, A.** 1979. Population changes induced in *Candida albicans* by nalidixic acid. Mycopathologia **88**:105–120.
18. **Silberstein, G. B., and A. B. Hooper.** 1975. The effect of the herbicide 2, 4, 5 trichlorophenoxy acetic acid (245T) on the growth and metabolism of *Tetrahymena pyriformis*. J. Cell. Physiol. **85**:331–338.